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Molecular Analysis of GB Virus C Isolates in Belgian Hemodialysis Patients

Hsin-Fu Liu,[1] Chantal Cornu,[1] Michel Jadoul,[2] Karin Dahan,[3] Guy Loute,[4] and Patrick Goubau[1]*

[1]*Unit of Virology, Cliniques Universitaires St-Luc, Université Catholique de Louvain, Brussels, Belgium*
[2]*Unit of Nephrology, Cliniques Universitaires St-Luc, Université Catholique de Louvain, Brussels, Belgium*
[3]*Unit of Genetics, Cliniques Universitaires St-Luc, Université Catholique de Louvain, Brussels, Belgium*
[4]*Department of Nephrology, St-Ode Central Hospital, St-Ode, Belgium*

GB virus C (GBV-C) has been detected in Belgian hemodialysis patients. To study their genomic diversity and phylogenetic relationship, a 592 nucleotide fragment extending from the 5′ noncoding region to part of the E1 gene of the GBV-C genome was amplified and sequenced from 12 Belgian hemodialysis patients in two different centers. Together with strains from different geographical origins, these sequences were analyzed phylogenetically using three different methods. A consistent tree topology was obtained with all methods. Three GBV-C genotypes were observed with two subtypes in type 2 and a questionable subtyping in type 1. Except for one isolate falling into type 1 cluster which mainly consists of African strains, all the other Belgian strains clustered within the type 2a branch. Two GBV-C isolates in two patients from the same hemodialysis center clustered together closely, suggesting a nosocomial transmission. In view of their long branch length, it seems unlikely that the other Belgian strains evolved recently from a common ancestor. Our results indicate that the major type circulating among Belgian hemodialysis patients seems to be 2a, which is usual for Europe and North America, but that the African type 1 also exists to a minor extent. Although patient to patient transmission of GBV-C in Belgian hemodialysis centers did occur, it may not account for the majority of infections. ***J. Med. Virol. 55:118–122, 1998.***

KEY WORDS: GB virus C; hepatitis G virus; phylogenetic analysis; 5′ noncoding region

INTRODUCTION

During the search for a possible etiological agent of non-A to -E hepatitis, the GB virus C (GBV-C), an enveloped positive single-stranded RNA virus with a genome length of about 9,400 nucleotides, has been identified in serum of a west African individual [Simons et al., 1995]. In an independent search, another flavivirus-like agent associated with chronic hepatitis was identified and designated hepatitis G virus (HGV) [Linnen et al., 1996]. Sequence comparison showed that GBV-C and HGV have approximately 86% nucleotide sequence and up to 95% amino acid similarity. They are thus presumed to be different isolates of the same virus [Zuckerman, 1996]. They both belong to the Flaviviridae family, which also includes the hepatitis C virus (HCV) [Choo et al., 1989]. Although the overall genomic structure of GBV-C and HGV is similar to that of HCV, they have only about 29% amino acid identity and therefore GBV-C and HGV are clearly distinct from HCV [Leary et al., 1996]. Unlike HCV, which can cause chronic hepatitis, cirrhosis, and possibly hepatocellular carcinoma [Choo et al., 1989; Saito et al., 1990], the clinical significance of GBV-C infection and its pathogenic role in hepatitis or any other disease remain unclear.

At least three major genotypes of GBV-C have been observed based on the sequence analysis of the 5′ noncoding region (5′NCR). It is predictive of the classification based on the complete genome [Smith et al., 1997]. Attempts to perform genotyping using the other genomic regions (e.g., NS3 helicase gene and NS5 fragment) were all unsuccessful [Kao et al., 1996; Tsuda et al., 1996; Pickering et al., 1997; Viazov et al., 1997]. Type 1 is more prevalent in West Africa, type 2 in Europe and North America, and type 3 in Asia [Muerhoff et al., 1996]. In addition, type 2 can be further divided into 2a and 2b, but the subtyping of type 1 is less certain. However, several studies have shown this geographical grouping is not absolute [Muerhoff et al., 1977; Smith et al., 1997]. The type 2 contains not only the European and North American isolates, but also strains from

*Correspondence to: Patrick Goubau, Unit of Virology, Cliniques Universitaires St-Luc, Université Catholique de Louvain, B-1200 Brussels, Belgium. E-mail: goubau@mblg.ucl.ac.be

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Pakistan, Japan, and East Africa. Although it has been suggested that a Chinese sequence might represent a distinct fourth genotype [Smith et al., 1997], a recent study indicated that this strain appeared to be a recombinant between type 3 and subtype 2a strains [An et al., 1997].

Hepatitis B and C viruses are major infective agents in hemodialysis patients. Not surprisingly, infection with GBV-C has indeed also been reported in maintenance hemodialysis [Beccari et al., 1996; Masuko et al., 1996]. We have detected GBV-C RNA in Belgian hemodialysis patients with an average prevalence of 16% [Cornu et al., 1997]. To clarify their genomic diversity and phylogenetic relationship, phylogenetic analysis was carried out on the 5′NCR of the viral genome in 12 GBV-C RNA positive patients from two different hemodialysis centers.

MATERIALS AND METHODS

Serum Samples

GBV-C RNA was detected in 11/78 (14.1%) hemodialysis patients from a Brussels center and 8/41 (19.5%) patients from a center in the Ardennes. A total of 12 positive serum samples, 9 (RM, DJ, WY, WA, VC, PA, AM, DR, DH) from patients of which sufficient serum was available in the Brussels hemodialysis center and 3 (GP, MV, TJ) from the center in the Ardennes, taken randomly for comparison, were studied further.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Sequencing

RNA was extracted from 50 μl of serum using the guanidinium isothiocyanate-phenol-chloroform method and used for cDNA synthesis as described previously [Cornu et al., 1997]. Five microliters of the cDNA was used in a nested PCR amplifying the 5′NCR to part of the putative E1 gene of GBV-C genome. The outer and inner primer pairs are GNTR1 (5′ CCG GCA CTG GGT GCA AGC CC 3′) with GNTR2 (5′ CCA/G CCC/T TCI AGG CAG AAC CC 3′) and S1 (5′ CAC TGG GTG CAA GCC CCA GAA 3′) [Muerhoff et al., 1996] with GE1WB2 (5′ C/TA/GG GGC A/GCA ACA A/GTT TGT GAG 3′), respectively. PCR was performed under standard reagent conditions: a 50 μl of PCR 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, a 200 μM concentration of each dNTP, a 1 μM concentration of each primer, and 1.25 units of *Taq* polymerase (AmpliTaq, Perkin-Elmer, Oak Brook, IL). Amplification was carried out in a Gene Amp PCR system 9600 (Perkin-Elmer) with 40 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec. Two microliters of the outer amplification product was used for the inner PCR under the same cycling condition. A PCR fragment with the expected size of 630 bp was separated on a 2% agarose gel (SeaKem, FMC Bioproducts, Rockland, ME) and purified with QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA). The purified DNA was direct sequenced with primers S1, GNTR3 (5′ AAT GC/TC TCT CCT GA/GC CAA TA 3′), GNTR4 (5′ ACT GGC/T CIT TGC/T CAA CTC GC 3′), and GE1WB2 using the ABI PRISM Dye Terminator Cycle sequencing Core Kit on an ABI 373A DNA Sequencer (Perkin-Elmer) according to the manufacturer's instructions.

Phylogenetic Analysis

A Basic Local Alignment Search Tool (BLAST) [Altschul et al., 1990] search was carried out to obtain the other GBV-C sequences in the same amplified region available in the EMBL/GenBank data base. A total of 56 sequences were then aligned with the GeneWorks software (Intelligenetics, Geal, Belgium) followed by minimal manual editing. Phylogenetic construction and evaluation were undertaken using the Phylip (version 3.572) software package [Felsenstein, 1989], with the neighbor-joining (NJ) method, the Fitch and Wagner parsimony (pars) method, and the maximum likelihood (ML) method. The transition and transversion ratio was scored at about 2.3 by the MacClade (version 3.04) software package (Sinauer Associates) and was used with the Felsenstein [1989] model to calculate the evolutionary distances. The robustness of the NJ and pars trees was statistically evaluated by bootstrap analysis with 1,000 bootstrap samples [Felsenstein, 1985]. The values on the branches represents the percentage of trees for which the sequences at one end of the branch are a monophyletic group. Since the ML method is already a statistical method (with a statistical evaluation of the branch length), no bootstrapping was done for it.

RESULTS

The 12 GBV-C sequences reported in Belgian hemodialysis patients were deposited in the EMBL/GenBank data base and were assigned the accession numbers Y15255 to Y15266. Nucleotide sequence variability between these strains is from 0.3% to 12.5% (Table I). The strain GP is the most divergent one among the 12 Belgian strains with sequence divergence of 10.3% to 12.5%. The strains DJ and DR have up to 99.7% sequence similarity (2 nucleotides substitution in the 592 bp sequenced fragment). The average variability between other strains is from 2% to 5%.

A total of 56 GBV-C sequences (Table II) were included in the phylogenetic analysis based on the 592 nucleotide 5′NCR fragment. A consistent tree topology was obtained from all methods and three GBV-C genotypes were observed (Fig. 1). Although not absolute, types 1, 2, and 3 consist mainly of isolates from Africa, Europe and North America, and Asia, respectively. The appearance of two subtypes (a and b) in type 2 was well supported by all methods, whereas the subtyping in type 1 is questionable. The ML tree provided strong support for subtypes 1a and 1b ($P < 0.01$) and the NJ tree also tended to separate these two clusters (Fig. 1). However, the bootstrap values for 1a and 1b clusters are only 60.5% and 61.1%, respectively, for the NJ tree. In the pars tree, subtype 1a failed to form a monophyletic group but subtype 1b remained supported by 66% bootstrap replicates.

We attempted to include more GBV-C strains from different geographical regions in the phylogenetic analysis. However, many of the available sequences in

TABLE I. Sequence Divergence (%) of 5′NCR of GBV-C Strains in Belgian Hemodialysis Patients

	AM	DH	DJ	DR	GP	MV	PA	RM	TJ	VC	WA	WY
AM	0											
DH	2.9	0										
DJ	2.9	3.4	0									
DR	2.9	3.7	0.3	0								
GP	11.8	11.5	10.6	10.3	0							
MV	3.4	4.4	4.7	4.7	12.5	0						
PA	4.2	4.2	4.6	4.2	11.8	2.7	0					
RM	3.5	4.6	5.2	5.1	12.1	5.6	5.9	0				
TJ	2.5	3.5	3.7	3.4	12.3	4.2	3.7	5.1	0			
VC	2.5	2.9	3.7	3.5	10.6	4.1	3.9	4.1	3.7	0		
WA	1.7	3.0	3.2	3.5	12.0	4.4	5.1	3.6	2.9	3.0	0	
WY	3.0	3.4	4.9	4.9	11.5	3.9	4.9	3.7	4.2	2.0	3.0	0

the 5′NCR are less than 400 nucleotides. Phylogeny reconstructed from the shortened sequence alignment though separating the three major genotypes led to subtypes 1a and 1b falling into one cluster and the subtyping of 2a and 2b also received a weaker support (data not shown).

All the Belgian strains clustered within the type 2a branch, except one isolate (GP) falling into the type 1b cluster (Fig. 1). The strains DR and DJ from the same hemodialysis center clustered together closely (98.6% for NJ, 94.9% for pars, and $P < 0.01$ for ML) within the 2a clade. The other Belgian GBV-C strains were interspersed between other subtype 2a isolates with relative long branch length compared to that of DR and DJ.

DISCUSSION

A prevalence of at least 16% of GBV-C viremia in Belgian hemodialysis patients was reported previously [Cornu et al., 1997]. No particular disease or hematological abnormality was found in these GBV-C RNA positive patients' files. Furthermore, the mean alanine aminotransferase levels did not differ between GBV-C positives and negatives, but were higher in the HCV infected [Cornu et al., 1997].

Although GBV-C infection seems widespread in hemodialysis patients, their phylogenetic relationship is not well understood. In the present study, we investigated the genomic diversity and phylogenetic relationship of 12 of the 19 GBV-C isolates in Belgian hemodialysis patients with other strains from different geographical locations. The analysis was based on the 5′NCR to part of the E1 gene of the viral genome because this region is more informative for phylogenetic analysis. Since the algorithms for constructing phylogenetic trees of different methods are based on different evolutionary assumptions, a consistent topology with all methods increases its reliability. We therefore performed three different types of phylogenetic analysis using the NJ, pars, and ML methods to increase the reliability of the derived topologies. The widely accepted bootstrap analysis was used for testing the robustness of the trees.

A consistent tree topology was obtained by all methods and three GBV-C genotypes were observed. The three major genotypes and the subtyping of 2a and 2b were well supported by all methods and were concordant with the results of others [Muerhoff et al., 1997; Smith et al., 1997], but the subtyping in type 1 is questionable. The branching patterns were inconsistent in different methods. The bootstrap values for separating subtypes 1a and 1b were only around 61% in the NJ tree and subtype 1a failed to form a monophyletic group in the pars tree. The robustness of the tree topology can be questioned since bootstrap values lower than 75% are generally not confident enough to fully support a topology [Zharkikh and Li, 1992]. In spite of this, the ML tree provided strong support ($P < 0.01$) for subtypes 1a and 1b. This would suggest a possible subtyping in GBV-C type 1 with a statistically significant support. Different genotypes might have diagnostic and clinical implications (e.g., use for assessing responses to antiviral therapy and future vaccine development) as suggested in the HCV-infected cases [Bukh et al., 1995; Gonzalez-Peralta et al., 1996].

In order to include more GBV-C strains from different geographical regions in the phylogenetic analysis, a shortened sequence alignment had to be used since a lot of available sequences in the 5′NCR are much shorter than ours. This led to subtypes 1a and 1b falling into one cluster and to a weaker support for the subtyping of 2a and 2b. Similar results have also been observed by others [Muerhoff et al., 1997]. By reducing the sequence length one apparently loses some important phylogenetic information which is necessary for separating the subtypes in GBV-C type 1.

Two GBV-C genotypes were found in Belgian hemodialysis patients. All the Belgian strains are type 2a, except the isolate GP, which belongs to type 1. The major type circulating in Belgium seems to be 2a, which is usual for Europe and North America, but the African type 1 also exists to a minor extent. As patient GP had no known African contacts, the GBV-C type 1 may have been present in Belgium for a certain period.

The GBV-C isolates from patients DR and DJ from the same hemodialysis center clustered together closely within the 2a clade and have up to 99.7% sequence similarity. This would imply a common source of infection. Indeed these two patients were dialyzed in

TABLE II. Origin of the GBV-C Isolates (in Alphabetical Order)

Strain	Geographical origin	Accession number
AM	Belgium	Y15257
DH	Belgium	Y15255
DJ	Belgium	Y15256
DR	Belgium	Y15259
GBV-C (EA)	East Africa	U63715
GBV-C1	United States	U59518
GBV-C2	United States	U59519
GBV-C3	Greece	U59520
GBV-C4	Italy	U59521
GBV-C6	United States	U59523
GBV-C7	United States	U59524
GBV-C8	United States	U59525
GBV-C9	United States	U59526
GBV-C10	United States	U59527
GBV-C11	United States	U59528
GBV-C12	Greece	U59529
GBV-C13	Greece	U59530
GBV-C14	United States	U59531
GBV-C15	United States	U59532
GBV-C16	Europe	U59533
GBV-C17	Greece	U59534
GBV-C18	United States	U59535
GBV-C20	United States	U59537
GBV-C22	Japan	U59539
GBV-C23	Ghana	U59540
GBV-C24	Ghana	U59541
GBV-C25	Ghana	U59542
GBV-C26	Ghana	U59543
GBV-C27	Ghana	U59544
GBV-C28	Ghana	U59545
GBV-C29	Ghana	U59546
GBV-C31	Ghana	U59547
GBV-C32	Ghana	U59548
GBV-C33	Ghana	U59549
GBV-C34	Ghana	U59550
GBV-C35	Ghana	U59551
GBV-C36	Ghana	U59552
GBV-C37	Ghana	U59553
GBV-C38	Ghana	U59554
GBV-C39	Ghana	U59555
GBV-C40	Ghana	U59556
GBV-C41	Ghana	U59557
GBV-C42	Ghana	U59558
GP	Belgium	Y15261
GS185	Japan	D87262
GT110	Japan	D90600
GT230	Japan	D90601
HGV-lw	Japan	D87255
MV	Belgium	Y15258
PA	Belgium	Y15260
PNF2161	United States	U44402
RM	Belgium	Y15262
TJ	Belgium	Y15263
VC	Belgium	Y15264
WA	Belgium	Y15265
WY	Belgium	Y15266

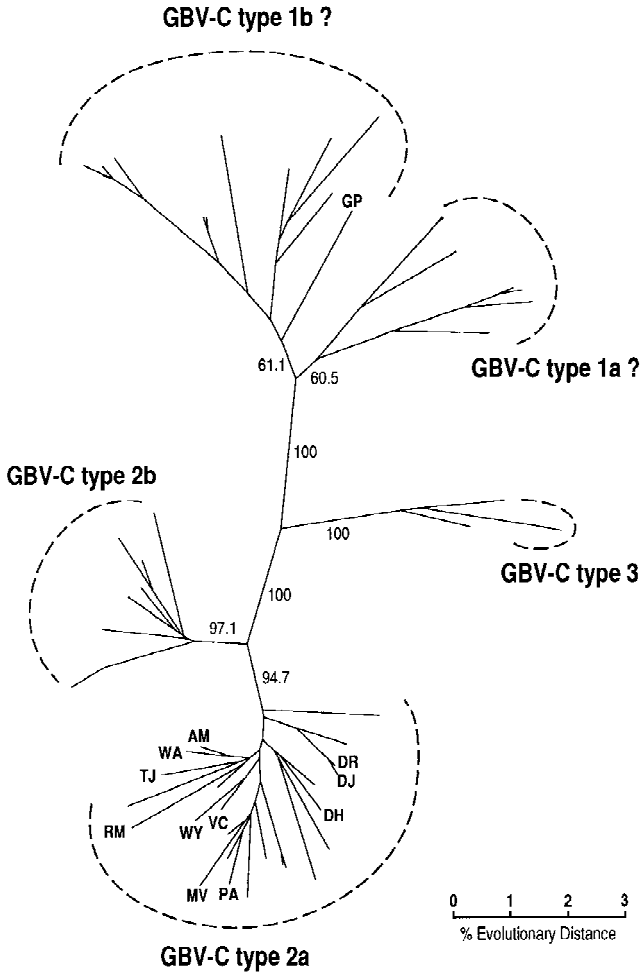

Fig. 1. Unrooted phylogenetic tree of GBV-C isolates from different geographical regions based on the 592 nucleotide fragment extending from 5′NCR to part of the E1 gene using the NJ method. The bootstrap values (1,000 bootstrap samples) are indicated beside the branches in percentage. Only the names of the strains in Belgian hemodialysis patients are shown. The strains in the clusters (moving clockwise around the figure) are as follows: GBV-C type 1b strains are GBV-C35, GBV-C33, GBV-C34, GBV-C36, GBV-C32, GBV-C37, GBV-C38, GBV-C31, GBV-C42, GBV-C41, GBV-C40, GBV-C39; GBV-C type 1a strains are GBV-C24, GBV-C25, GBV-C23, GBV-C28, GBV-C29, GBV-C27, GBV-C26; GBV-C type 3 strains are GBV-C22, GT230, GS185; GBV-C type 2a strains are GBV-C1, GBV-C2, GBV-C6, GBV-C4, GBV-C3, GBV-C10, GBV-C11, GBV-C9, GBV-C7, PNF2161, HGV-lw, GBV-C8, GT110; and GBV-C type 2b strains are GBV-C13, GBV-C12, GBV-C14, GBV-C16, GBV-C (EA), GBV-C15, GBV-C18, GBV-C20, GBV-C17.

adjacent beds in the same shift for a long period of time, a well-known risk factor for the nosocomial transmission of blood-borne viruses [Jadoul et al., 1993]. One (DR) of the two became infected between 1992 and 1993, while the other (DJ) was already infected in 1991. This indicates that patient DR was most probably infected from DJ. The other Belgian GBV-C strains were interspersed between other subtype 2a isolates with relative long branch length compared to that of DR and DJ. Based on this result, they are unlikely to have evolved recently from a common ancestor. Thus patient to patient transmission of GBV-C in Belgian hemodialysis centers did occur but could not account for the majority of infections.

In this study, it was shown that two GBV-C genotypes were present in Belgian hemodialysis patients. The major type seems to be 2a, but the African type 1 also exists. In the hemodialysis centers, patient to patient transmission occurred but may not be the main mode of GBV-C infection. The inconsistency of subtyping in type 1 by different phylogenetic analysis methods needs to be further studied.

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